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Center for Veterinary Biologics
Testing Protocol

SAM 322

Supplemental Assay Method for Determination of the
Specific Viral Antigen Content in Inactivated Canine
Coronavirus Vaccines

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1. Introduction

This Supplemental Assay Method (SAM) describes an enzyme-linked immunosorbent assay (ELISA) method for the quantitation of the viral antigen content of inactivated canine coronavirus (CCV) vaccines. The relative potency (RP) of CCV vaccines is determined by comparing the amount of CCV in a Test Vaccine to the CCV content of a Reference Preparation that has been shown, directly or indirectly, to be protective in a host animal immunogenicity study.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Incubator, 36° ± 2°C, 5 ± 1% CO₂, high humidity (Model 3158, Forma Scientific Inc.)

2.1.2 Microplate reader (Model MRX, Dynex Technologies Inc.)

2.1.3 Microplate washer (Model EL404, Bio-Tek Instruments Inc.)

2.1.4 Micropipettors: 200-µl and 500-µl single channel, 50-200 µl x 12-channel, and tips

2.1.5 Microtiter plate shaker (Model 4625, Labline Instruments Inc.)

2.1.6 RP Calculation Method: Current version of *Supplemental Assay Method for Evaluation by the Relative Potency Method of In Vitro Enzyme Immunoassays Used In Testing of Veterinary Vaccines* (SAM 318)

2.1.7 Current version of the U.S. Department of Agriculture, Center for Veterinary Biologic's (CVB) *Relative Potency Calculation Software (RelPot)* [available from CVB]

2.1.8 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

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2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 0.01 M Phosphate buffered saline (PBS)

2.2.1.1 1.9 g sodium phosphate, dibasic,
anhydrous (Na_2HPO_4)

2.2.1.2 0.22 g sodium phosphate, monobasic,
monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)

2.2.1.3 8.5 g sodium chloride (NaCl)

2.2.1.4 Q.S. to 100 ml with distilled water (DI).

2.2.1.5 Adjust pH to 7.2-7.6 with 0.1 N sodium
hydroxide (NaOH) or 1.0 N hydrochloric acid (HCl).

2.2.1.6 Sterilize by autoclaving at 121°! 2°C,
15 psi for 35 ! 5 minutes.

2.2.1.7 Store at 2°- 7°C.

2.2.2 0.05 M Carbonate/Bicarbonate Coating Buffer, pH 9.6

2.2.2.1 0.159 g sodium carbonate (Na_2CO_3)

2.2.2.2 0.293 g sodium bicarbonate (NaHCO_3)

2.2.2.3 Q.S. to 100 ml with DI.

2.2.2.4 Adjust pH to 9.6 with 2 N HCl .

2.2.2.5 Store at 2°- 7°; use within 1 week.

2.2.3 Blocking Solution

2.2.3.1 1 g casein in 100 ml Carbonate Coating
Buffer

2.2.3.2 Store at 2°- 7°; use within 1 week.

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2.2.4 Wash Buffer

2.2.4.1 500 µl Tween-20 in 1000 ml 0.01 M PBS

2.2.4.2 Store at room temperature.

2.2.5 Diluent Buffer

2.2.5.1 1 g casein in 100 ml Wash Buffer

2.2.5.2 Store at 2°- 7°C; use within 1 week.

2.2.6 Feline infectious peritonitis virus antibody (FIPV Ab), ammonium sulfate-precipitated [available from CVB]

2.2.7 CCV monoclonal antibody (CCV MAb) [available from CVB]

2.2.8 Rabbit anti-mouse horseradish peroxidase conjugate (Rabbit Anti-mouse Conjugate)

2.2.9 (2,2'-azino-di-{3 ethyl-benzthiazaline sulfonate 6}) (ABTS) peroxidase substrate solution (Substrate Solution)

2.2.9.1 Solution A, ABTS

2.2.9.2 Solution B, Hydrogen Peroxide

2.2.10 Flat bottom, 96-well ELISA plate (ELISA Plate)

2.2.11 Plate sealer

2.2.12 Reference Preparation. Each manufacturer provides a Reference Preparation that has been directly or indirectly shown to be protective in a host animal immunogenicity trial. The Reference Preparation is the lot number identified in Part V of the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production or special outline. All subsequent serials produced by a manufacturer must have an RP equal to or greater than the RP value contained in the APHIS filed Outline of Production.

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3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training and experience in the immunological basis of antigen capture ELISA assays, the principles of optical densitometry (OD), and computer software analysis.

3.2 Preparation of equipment/instrumentation

The microplate reader must be turned on at least 30 minutes prior to determination of OD readings. The microplate reader is zeroed on air prior to initial use.

3.3 Preparation of reagents/control procedures

3.3.1 Test Plate preparation. On the day of plate coating, dilute the FIPV Ab, per the Center for Veterinary Biologics (CVB) Reagent Data Sheet supplied with the reagent, in Carbonate/Bicarbonate Coating Buffer. Mix by vortexing and pipette 100 µl of diluted FIPV Ab to each well of an ELISA Plate, which becomes the Test Plate. Cover the Test Plate with a plate sealer and incubate 3 ! 2 days, at 2°- 7°C.

3.3.2 CCV MAb preparation: On the day the Test Plate is read, dilute the CCV MAb, per the CVB Reagent Data Sheet supplied with the reagent, in Diluent Buffer; mix by vortexing.

3.3.3 Rabbit Anti-Mouse Conjugate preparation: On the day the Test Plate is read, dilute the Rabbit Anti-mouse Conjugate, per previously determined optimal dilution, in Diluent Buffer; mix by vortexing.

3.3.4 Substrate Solution preparation. On the day the Test Plate is read, just prior to substrate addition, mix equal volumes of ABTS Solution A and Hydrogen Peroxide Solution B, per the manufacturer's instructions. The resulting Substrate Solution must remain clear. The Substrate Solution must be at room temperature at time of use.

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3.4 Preparation of the sample

3.4.1 Antigen Extraction (Optional). If the Test Vaccine contains an adjuvant which interferes with antigen detection, the firm may specify the procedure for extraction of the antigen from the adjuvant. If extraction is a necessary step, the extraction procedure will be included in Part V of the APHIS filed Outline of Production or special outline. If the Reference Preparation is a product reference, both the Reference Preparation and Test Vaccine must be treated identically. If the Reference Preparation is a purified reference, the extraction procedure is not required for the Reference Preparation. The CVB will extract antigen using the firm's protocol. If no protocol is stated in either the APHIS filed Outline of Production or special outline, the test will be conducted at the CVB without extraction.

3.4.2 All samples must be at room temperature before testing. The initial test of a Test Vaccine will be with a single vial (a single sample from 1 vial). Twofold dilutions may be made in an additional ELISA Plate, which becomes the Transfer Plate, as follows (tips are changed between each dilution):

3.4.2.1 Add 150 µl of Diluent Buffer to wells in rows B-H with a 12-channel micropipettor (see **Section 9.1**).

3.4.2.2 The starting dilution for the Reference Preparation and the Test Vaccine shall be stated in part V of the APHIS filed Outline of Production or special outline. Unless stated otherwise, the diluent will be Diluent Buffer for the initial dilution.

3.4.2.3 Add 300 µl of the starting dilution of the Reference Preparation to wells A1 and A2.

3.4.2.4 Add 300 µl of the starting dilution of the Test Vaccine to wells A3 and A4. Additional Test Vaccines may be tested in duplicate in columns 5-12.

3.4.2.5 Transfer 150 µl from row A to row B. Mix row B with the 12-channel micropipettor (7 ! 2 fills).

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3.4.2.6 Continue as in **Section 3.4.2.5** for the remaining rows C-G, transferring 150 µl from the previous row to the next row. Note: Row H is not used and remains as Diluent Buffer for transfer to the blank wells.

4. Performance of the test

4.1 Remove the Test Plate prepared earlier from 2°- 7°C storage (see **Section 3.3.4**).

4.2 Decant the Test Plate contents in a suitable container. Fill each well with at least 200 µl of Wash Buffer. Immediately decant Wash Buffer from the Test Plate. Repeat for a total of 4 washes. At no time should wells dry between rinses or incubations. After the last wash, tap the Test Plate on paper towels to remove residual Wash Buffer. An automatic plate washer may be used.

4.3 Add 200 µl of Blocking Solution to each well of the Test Plate; seal the Test Plate with a plate sealer and incubate for 60 ! 10 minutes at 36° ! 2°C.

4.4 Wash the Test Plate as in **Section 4.2**.

4.5 Transfer 100 µl of the Test Vaccine and the Reference Preparation diluted on the Transfer Plate (see **Section 3.4.2**) to corresponding wells of the blocked Test Plate. Tips need not be changed if proceeding from the most dilute to the most concentrated (Row H to Row A). Row H receives all assay reagents except antigen and serves as the blank wells.

4.6 Seal the Test Plate with a plate sealer and incubate on the microtiter plate shaker 2 hours ! 10 minutes, or overnight (14 ! 2 hours), at 36°! 2°C with sufficient agitation to keep the test samples in suspension. The incubation time shall be stated in Part V of the APHIS filed Outline of Production or special outline. If the incubation time is not specified, incubation will be overnight.

4.7 Wash the Test Plate as in **Section 4.2**.

4.8 Pipette 100 µl/well of Diluted CCV MAb to each well of the Test Plate; seal the Test Plate with a plate sealer and incubate for 60 ! 10 minutes at 36°! 2°C.

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4.9 Wash Test Plate as in **Section 4.2**.

4.10 Pipette 100 µl of Diluted Rabbit Anti-mouse Conjugate to each well of the Test Plate; seal the Test Plate with a plate sealer and incubate for 60 ± 10 minutes at 36° ± 2°C.

4.11 Wash the Test Plate as in **Section 4.2**.

4.12 Pipette 100 µl of Substrate Solution to each well of the Test Plate; incubate the Test Plate at room temperature.

4.13 Read the Test Plate at 405-nm test wavelength against a 490-nm reference wavelength on the microplate reader when the color development gives a sufficient OD reading in at least the fourth dilution of the Reference Preparation (OD ≥ 0.05 after the average blank reading is subtracted).

4.14 Determine the arithmetic mean of at least 3 wells of the blank wells in Row H; this becomes the Average Blank Reading. The Average Blank Reading is subtracted from all readings before analysis of the data by *RelPot*.

4.15 Evaluate the data using *RelPot*.

5. Interpretation of the test results

5.1 All validity criteria in the current version of **SAM 318** must be met for a valid test. An invalid test may be repeated. Testing may be repeated for equivocal tests as defined in 9 CFR 113.8(c)(4).

5.2 For a Test Vaccine to be satisfactory, the RP value of at least 1 valid RP from the group of the highest scoring valid RP values has to be greater than or equal to the RP stated in an APHIS filed Outline of Production.

5.3 For a Test Vaccine with an RP value less than the RP stated in an APHIS filed Outline of Production, the test may be repeated when the test meets the criteria defined in 9 CFR 113.8(c)(5) (see **SAM 318**, Test Results and Interpretation).

6. Report of test results

Record RP results on the test record.

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7. References

7.1 Code of Federal Regulations, Title 9, U.S. Government Printing Office, Washington, DC, 2004.

7.2 Horsburgh BC, Brown TDK. Sequence analysis of CCV and its relationship to FIPV, TGEV and PRCV. In: *Coronaviruses*, Laude H, Vautherot J, eds., pp 3-9. Plenum Press, New York, 1994.

7.3 Katz JB, Hanson SK, Patterson PA, Stoll IR. *In vitro* assessment of viral antigen content in inactivated aluminum hydroxide adjuvanted vaccines. *J Vir Meth* 1989, 25:101-108.

8. Summary of revisions

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- The refrigeration temperatures have been changed from $4^{\circ} \pm 2^{\circ}\text{C}$ to $2^{\circ} - 7^{\circ}\text{C}$. This reflects the parameters established and monitored by the Rees system.
- "Test Serial" has been changed to "Test Vaccine" throughout the document.
- The footnotes have been deleted with any pertinent references now noted next to the individual items.
- "Reference and Reagent Sheet" has been changed to "Reagent Data Sheet" throughout the document.

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9. Appendix I: Transfer and Test Plate Format

	1	2	3	4	5	6	7	8	9	10	11	12
A	REF	REF	TS1	TS1	TS2	TS2	TS3	TS3	TS4	TS4	TS5	TS5
B												
C												
D												
E												
F												
G												
H	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK	BLK

REF= Reference Preparation; TS= Test Serial; BLK= Blank